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HISTOCHEMICAL STUDIES OF ALKALINE PHOSPHATASE IN THE OOGENESIS AND THE EARLY EMBRYOGENESIS OF THE AMPHIBIA*

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INTRODUCTION

Since the publication of a histochemical technique for demonstration of the alkaline phosphatase by TAKAMATSU (1938, 1939) and GOMORI (1939, 1941), numerous studies have been made to localize the enzyme within various tissues. The importance of this enzyme during embryogenesis was emphasized for the first time by MOOG (1944, 1946), who studied it in chick embryo with histochemical and biochemical method, and concluded that this enzyme is closely related to the differentiation. Subsequently, BRACHET (1946) pointed out that in amphibian embryo the phosphatase reaction is weak during the early stages, but becomes intensive at the beginning of differentiation when the synthesis of new protein at the expense of yolk material is supposed to be active. Moreover, KRUGELIS (1947) showed in a brief note that in the amphibian blastula the reaction in the nucleus is very weak showing no regional difference, but during gastrulation the enzyme is synthesized both in the nucleus and cytoplasm: its reaction is stronger in the ectoderm than in the mesoderm and is very faint in the endoderm. This difference becomes more distinct in the neurula stage. The localization of the enzyme in the cytoplasm closely parallels with that of ribonucleoprotein observed by BRACHET (1942). Those facts lead BRACHET (1947 b, 1949, 1950) to assume that the cytoplasmic alkaline phosphatase is synthesized as a component of the basophilic granules of ribonucleoprotein which are distributed in accordance with the morphogenetic gradient, and whose importance in the morphogenesis was repeatedly stressed by himself (1947 a, 1949, 1950). BRACHET and JEENER (1948) found that the alkaline phosphatase in the nucleus of the adult rat tissues is related to the turnover of phosphorus of desoxyribonucleic acid. Citing this fact and the KRUGELIS' data, BRACHET (1947 b, 1949) supposed that even in the gastrula stage, the differentiation of the nuclei of different embryonic regions already occurs as to the renewal of phosphorus in the desoxyribonucleic acid.

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Inspired by the works of CASPERSSON (*cf.* CASPERSSON, 1950) and BRACHET (*cf.* BRACHET, 1947 a) numerous attempts have been made with success to establish the close parallelism between the nucleic acids content and the intensity of the protein synthesis in cells. It is now generally recognized that the phosphate bonds play an important role in the synthetic processes including protein formation. From this stand point, SPIEGELMAN and KAMEN (1946, 1947) suggested a possibility that nucleoproteins are specific energy donors for protein synthesis. If the latter process indeed involves phosphorylation and dephosphorylation reactions, a significance must be attached to the enzyme such as alkaline phosphatase. In fact, many instances are known where phosphatase is particularly rich in cells which are engaged in protein synthesis (*cf.* DAVIDSON, 1949). For example, BRADFIELD (1946, 1949) and JEENER (1948) clearly showed that the ability to synthesize the fibrous protein is always associated with the presence of a large amount of alkaline phosphatase. The problem therefore arises whether the sites of phosphatase activity are closely linked up with that of nucleoproteins. Many authors showed that this is the case. Thus, it might be expected that in the early amphibian embryos alkaline phosphatase participates in the synthesis of protein particularly of the fibrous type, in collaboration with nucleic acids.

However, in the course of histochemical studies on the alkaline phosphatase of tissues, GOMORI's technique has been severely critisized. Several authors have pointed out the possibility of artefact due to diffusion of enzyme or/and of reaction product during fixation and incubation which might occasionally lead us into pitfall (*cf.* LISON, 1948; RUYTER and NEUMANN, 1949; DANIELLI, 1950; FEIGIN, WOLF and KABAT, 1950; GOMORI, 1950 b; JACOBY and MARTIN, 1950; NOVIKOFF, 1951; YOKOYAMA, STOWELL and MATHEWS, 1951). Because of this reason, it was thought worthwhile to reexamine the foregoing reports critically and to forward our knowledges as to the distribution and possible role of the alkaline phosphatase in the amphibian eggs and embryos. In the present study the localization of alkaline phosphatase in the oogenesis of *Triturus pyrrhogaster* and in the embryogenesis of *Hynobius tokyoensis* and *Rhacophorus schlegelii*, var. *arborea* was studied with histochemical technique which was examined critically, and the possible role of the enzyme in the developmental processes was considered afresh.

MATERIALS AND TECHNIQUES

1. *Biological.* The ovarian eggs of newt, *Triturus pyrrhogaster*, and the embryos of various developmental stages of green frog, *Rhacophorus schlegelii*, var. *arborea* and a salamander, *Hynobius tokyoensis* were used as materials. The unpigmented eggs of green frog were found very suited for the histochemical study.

The developmental stages of *Hynobius tokyoensis* and *Rhacophorus schlegelii*, var. *arborea* in the present study were classified according to USUI and HAMAZAKI's[1] (1939) and ICHIKAWA's table (1931) respectively. Following stages were

[1] This table is for *Hynobius nigrescens*, but the development of *tokyoensis* is closely similar with that of *nigrescens*.

used:

a. *Hynobius tokyoensis.* St.[2] 11 (early gastrula); St. 12 (late gastrula); St. 17 (mid-neurula); St. 22' (tail bud stage I, text-fig. 1); St. 25 (tail bud stage II, text-fig. 2); St. 33 (tail bud stage III, text-fig. 3); St. 36 (tail bud stage IV, text-fig. 4).

b. *Rhacophorus schlegelii,* var. *arborea.* St. 0 (uncleaved fertilized egg); St. 6 (morula); St. 8 (early gastrula); St. 13 (late gastrula); St. 24 (neurula); St. 31 (tail bud stage, text-fig. 6); St. 44 (embryo before hatching, text-fig. 7); St. 47 (newly hatched embryo, text-fig. 8); Swimming larva (one and half months after hatching).

2. *Histochemical.* The histochemical method used for detection of alkaline phosphatase was that developed by TAKAMATSU (1938, 1939) and GOMORI (1939) with minor modification. Fresh ovary containing oocytes of various stages, and embryos were fixed in chilled acetone for 24 hrs. in a refrigerator or a vacuum-bottle at 0°~5°C. After fixation, the material was washed in acetone for 1 hr. at room temperature, then put in benzol for 30 min. and embedded in paraffin (52°C., 1~1.5 hrs.). In some cases the embryos were fixed and embedded by freezing-drying technique employing the apparatus constructed after NAKASHIMA, TSUJII and NAORA (1950). Section was cut 15 μ.

The sections attached to slide with glycerine-egg albumin were deparaffinized in benzol, then carried through descending series of acetone (100% → 90% → 70%) to distilled water, and transfered to the substrate solution which was previously warmed to 37°C. in the incubator.

The substrate mixture was freshly prepared for each experiment according to the following formula:

0.1 M Sodium glycerophosphate	6 cc.
2% Sodium barbital (or M/10 glycine buffer, pH 9.4)	2 cc.
0.75% $CaCl_2$	5 cc.
5% $MgCl_2$	1 cc.
Distilled water	3 cc.

Before incubation pH of the mixture was checked using thymol blue and adjusted to 9.2 (or 9.4 when glycine buffer was used). The incubation lasted 30 min. to 72 hrs. according to circumstances at 37°C. For the visualization of the site of enzyme activity, VON KOSSA's procedure was empolyed: after removing from the incubation mixture, the slides were rinsed in distilled water, immersed in 5% silver nitrate solution and exposed to the sun light for 5~10 min. or to an electric lamp (200 W.) for 1 hr. The slides were then placed in 0.7% NaCl solution, rinsed in distilled water, and immersed in 5% sodium thiosulfate for 15 seconds. After rinsing in distilled water they were dehydrated, cleared and mounted following usual histological technique. The positive site was brown or black. In all cases controls were made to check the possible presence of preformed phosphate in the tissues and occurrence of non-specific deposition of silver nitrate. For this purpose slides were incubated in a solution without glycerophosphate, or heated in the boiling distilled water for

[2] The stage described in the tables.

5 min. before incubation and then placed in the substrate medium.

There is an alternative method for the final visualization of calcium phosphate caused by enzymatic reaction (GOMORI, 1941). The method involves the use of cobalt with the final visible product being cobalt sulfide. Most of the investigators in the field use this method. For amphibian eggs and embryos, however, this cobalt method is inappropriate because of the strong affinity of cobalt to yolk component or to nucleoli of oocytes: the control sections (the section of embryo or of ovary incubated in the substrate-free medium, previously heated section incubated in the substrate medium, or non-incubated deparaffinized section) treated with cobalt solution always showed a considerable non-specific deposition of cobalt on the above components which subsequently appeared as cobalt sulfide. Similar observations were reported by KRUGELIS (1946) and NEWMAN *et al.* (1950) for other cell structures of some tissues. Not only in the adult tissues but also in the oocytes or embryos it is possible that the enzyme diffuses out during fixation from the original site to surrounding regions or during incubation diffusion of the enzyme or/and calcium phosphate occurs and the diffusing substance(s) is taken up selectively by some cellular structures. Several series of experiments were devoted to check the occurrence of diffusion, and thus it was attempted to determine the precise site of the enzyme activity. Techniques employed for this purpose will be detailed in each case..

RESULTS

1. *Oogenesis*

In the young oocytes (yolk granules not yet developed) incubated for shorter time (1 hr.) in the substrate medium, only the follicle layer was colored positively (pl. 1, fig. 1). After longer incubation (2 hrs.) some of the nucleoli of the young oocyte were stained beside follicle, and after still longer incubation (24 ~ 72 hrs.) follicle, nucleoli, nuclear sap were strongly stained, sometimes accompanied by weak coloration of the cytoplasm (pl. 1, fig. 1). The older the oocyte, the weaker the coloration in the germinal vesicle.

In the grown oocytes, the pattern was the same as that of the young ones if they were incubated for 1 hr., the positive site being only the follicle (pl. 1, fig. 1). However, after prolonged incubation of grown oocytes yolk granules were also stained (pl. 1, fig. 2), while no reaction was observed in the nucleus except in nucleoli which showed weak coloration in some cases.

The duration of incubation necessary for obtaining above mentioned positive reaction varied slightly according to circumstances such as the season of experiment or condition of the adult from which ovary was dissected out, although the order of reactivity among each structure was unchanged. Table I showes a typical case.

In view of the possibility of diffusion during incubation time, it seemed somewhat doubtful that all of the positive reactions observed in the oocytes after prolonged incubation were the real phosphatase reaction produced *in situ.* From this point of view, some experiments were carried out aiming to establish the true localization of enzyme activity in the oocytes.

Table I. The reactivity of some structures of the *Triturus* ovary treated by GOMORI's technique.

Incubation period	Staining reaction							pH of medium
	Young oocyte			Grown oocyte				
	Follicle	Nucleoli	Nuclear sap	Follicle	Nucleoli	Nuclear sap	Yolk granules	
0 hr.	−	−	−	−	−	−	−	9.2
1	+	−	−	+	−	−	−	9.2
2	+	−(or +)	−	+	−	−	−	9.2
3	+	+	+	+	−	−	+	9.0
6	+	+	+	+	−	−	+	8.9
12	+	+	+	+	−(or +)	−	+	8.8
24	+	+	+	+	−(or +)	−	+	8.6

Experiment 1. (Idea of DANIELLI (1946)). A deparaffinized section of ovary attached to slide was heated in the boiling distilled water for 5 min. to inactivate completely the enzyme. Such section was utterly unstainable when incubated alone even for 72 hrs. Now another section from the same material was deparaffinized and superimposed on the inactivated section. GOMORI's technique with varying incubation periods was then applied. Such procedure might reveal whether the boiled section is able to stain under the influence of the unboiled section. After 24 hrs. of incubation, in the underlying inactivated section the nuclear sap and nucleoli of young oocytes and yolk granules of grown ones gave a strong positive reaction (pl. 1, fig. 3). On the contrary, the egg follicle showed no or only weak color (pl. 1, fig. 3), although if the section were incubated alone without heat treatment, the follicle should be the first to stain most intensively after 24 hrs. incubation. From this result, it may be concluded that the above noted "positive reaction" in the underlying oocytes is a translocated manifestation of the enzyme or/and calcium phosphate of the overlying section. The facts suggest further that the diffusing enzyme or/and reaction product has a strong affinity with the nucleoli, a lesser one with the nuclear sap (of young oocytes) and yolk granules (of grown one), and the least one with the follicle.

Experiment 2. Paraffin section of the ovary was attached to slide according to the usual cytological technique. Before, or occasionally after, deparaffinization in benzol, the follicle of young or grown oocytes was removed completely as possible with aid of a fine steel needle under the binocular dissecting microscope. Slide with this operated oocytes was then treated according to GOMORI's method. As result, most of follicle-free oocytes of entire stages examined showed no evidence of reaction even after 24 hrs. incubation, whereas the non-operated oocytes developed an intense color in their nucleoli, nuclear sap (of young oocytes) and yolk (of grown ones) after the same incubation time. In very small number of cases (3 out of 22 cases), nucleoli, but not nuclear sap, colored slightly after 24～48 hrs. incubation.

Experiment 3. The egg portion was removed completely from paraffin section, leaving the isolated follicle. The latter was then incubated for 24 hrs. In all cases strong coloration was obtained in the follicle.

From the result of the three series of experiments mentioned above, it is obvious that the enzyme or/and the reaction product diffuses out from the egg follicle during incubation to be adsorbed from the nucleoli, nuclear sap or yolk granules, and the reaction in the latter three structures after long incubation is at least in most cases nothing but an artefact. It is not obvious whether above three positive cases of reaction on the nucleoli in *Experiment 2* is due to the presence of small fragment of active follicle layer or true enzyme activity associated with nucleoli themselves. If the positive color was due to the enzyme activity produced *in situ*, the color was extraordinary weaker than that obtained with the standard GOMORI's technique without additional operation. Thus the large part of the nucleolar staining observed without any supplementary operation is due to artefact caused by diffusion. It may be concluded that with GOMORI's cytochemical method combined with supplementary techniques, the true enzyme activity can be detected constantly only in the egg follicle throughout entire periods of oogenesis, although there remains possibility that an amount of enzyme present within egg cell proper was destroyed during successive histological treatment here adopted.

According to GOMORI (1950 a, b), the solubility of calcium phosphate produced by enzymatic reaction is exceedingly low at pH 9.0 to 9.2, thus no sign of diffusion could be noted when the test of DANIELLI's type was carried out at this pH range, but the diffusion increased rapidly with lowering of pH of the medium. This observation might suggest that also in our case the diffusion phenomenon is correlated to a drop of pH during incubation. In a series of experiments this point was put on test. A series of comparable slides was incubated for different duration and for each case the staining reaction of the nucleoli was observed as the measure of diffusion and pH of the solution was estimated. The results obtained were summarized in Table I. It seems probable, that occurrence of diffusion does not depend on pH drop of the incubation mixture, as the sign of diffusion began to occur even at the original pH level (pH 9.2). Therefore, from the above experiment, the cause of diffusion could not be cleared.

2. *Embryogenesis*

A. *Hynobius tokyoensis*

The silver precipitation was always observed to be limited to the yolk granules and the pseudopod-like cytoplasm of mesenchyme cells. As the cells of this species contain much black pigment, it is very difficult to detect the silver deposit even if it would present in the matrix of the cytoplasm. There is no conclusive evidence whether the silver precipitation in the yolk granules was produced by the enzymatic activity *in situ* or the activity was restricted to the cytoplasmic matrix and the diffusing substance was taken up selectively by the yolk granules, or both structures contain themselves the enzyme. In the present state, therefore we have no data to suggest as to the intra-cytoplasmic localization of the enzyme of this species. In contrast to the result of the earlier workers, the nucleus showed in most cases no silver precipitation, and

if any precipitation was detectable, it was very weak. This holds for the entire regions and stages of embryo here studied.

As in this series no experiment was performed to check the possible diffusion, the pattern of reaction described below may not always correspond precisely the primary pattern of the enzyme activity.

The incubation periods used in this series were 2, 4, 8 hrs. and in particular cases 22 hrs.

(a) St. 11 (early gastrula); St. 12 (late gastrula); St. 17 (mid-neurula): Enzyme activity could not be demonstrated in the above three stages, even if the reaction was allowed to proceed for 22 hrs.

(b) St. 22 (tail bud stage I, text-fig. 1): There was no evidence of the phosphatase activity on the section after 2 to 8 hrs. incubation. After 22 hrs., however, feeble but distinct silver precipitation was obtained only in some cytoplasmic parts of presumptive pronephric region and cutis layer in the same segment of the presumptive pronephros.

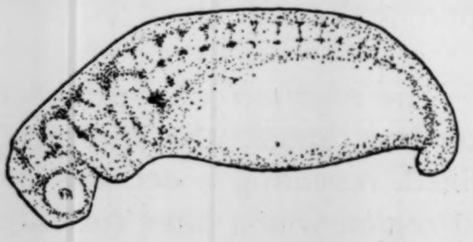
(c) St. 25 (tail bud stage II, text-fig. 2): The reaction was rather feeble in general, but in comparison with the preceding stage, the intensity was strengthened and the positive area was amplified, revealing a definite localization. The mesectodermal mesenchyme of head region which later forms principally the head cartilage was clearly reactive after 4 hrs. incubation, the remaining ectoderm being still negative. The pronephric region and cutis layer of the somite showed the strongest reaction of all the embryonic regions. The latero-ventral mesoderm reacted positively only in its anterior part. This positive mesodermal region might be the presumptive heart. Notochord and myotome were wholly negative. No positive site was observed also in the endoderm.

Text-fig. 1. *Hynobius tokyoensis*, St. 22 (tail bud stage I).

Text-fig. 2. *Hynobius tokyoensis*, St. 25 (tail bud stage II).

(d) St. 31 (tail bud stage III, text-fig. 3): In the embryonic parts, which were positive in St. 25, the reaction was increased considerably, and new positive regions were added. In this stage the mesectodermal cells were condensing in several regions of the head to form the rudiment of cartilage, such as MECKEL's cartilage, trabecula cranii, quadrate cartilage, basihyal, ceratohyal, and ceratobranchial. Both loose and packed mesenchyme cells in these regions were intensely stained in their cytoplasm. Particularly in the rudiment of MECKEL's cartilage the reaction was strong. The differentiating retina and the lens reacted weakly. Other ectodermal structures were wholly negative after 8 hrs. of incubation. The pronephros which was forming tubules stained strongly, the

reactivity being most intensive throughout the embryo. The heart rudiment in which the endocardium and pericardium were differentiating was found to have marked brown precipitation. The blood island reacted weakly in its anterior part but negatively in its posterior part. The cutis layer of the somite and the lateral plate, where the activity was insignificant in the preceding stage, showed stronger reaction in its anterior parts, thus revealing the gradient of intensity from anterior to posterior. The anterior segments (1~5 or 6) of somite stained considerably, but the more posterior myotomes did not stain at all. The cartilage forming mesodermal mesenchyme around the auditory vesicle reacted as strongly as the mesectodermal mesenchyme. The walls of pharynx and oesophagus, the liver region and the ventral periphery of the endodermal yolk mass stained weakly.



Text-fig. 3. *Hynobius tokyoensis*, St. 33 (tail bud stage III).

Text-fig. 4. *Hynobius tokyoensis*, St. 36 (tail bud stage IV).

(e) St. 36 (tail bud stage IV, text-fig. 4): The pattern of the reaction was essentially the same as the preceding stage, although its intensity was increased. Mesectodermal loose mesenchyme of the head region revealed strong positive reaction. The strongest reaction was observed in chondrogenetic mesenchyme cells in the rudiment of head cartilage, for instance in that of MECKEL's cartilage. Feeble reaction was also obtained in a part of forebrain and of neural tube. The remaining regions derived from the ectoderm was entirely negative. In the pronephros, which has formed wounded tubules, most intensive black precipitate was demonstrated even after 4 hrs. of incubation. The cutis layer, sklerotome and ventral process of the somites revealed strong positive reaction. The heart, mesodermal mesenchyme around the auditory capsule or in the tail bud showed strong phosphatase activity, while the blood island was weakly positive in its anterior, but negative in its posterior part. Anterior segments of the myotome showed considerable reaction. Remaining somite, notochord and anterior lateral plate also revealed light yellow precipitation. No marked change was ascertained as to the endoderm in comparison with St. 31. But entire endodermal yolk mass showed weak yellow color.

The foregoing results for *Hynobius* may be summaried as follows: Up to the late neurula stage the enzyme activity could not be found by means of histochemical technique here employed. At the earlier tail bud stage the first sign of enzyme reaction appeared very feebly in the presumptive pronephric region and the cutis layer of the somites. The reaction in the pronephric region increased with progress of its differentiation and reached the maximum at the stage when the pronephric tubules differentiated. Following the first

sign of pronephric enzyme activity, the reaction could be demonstrated in the mesectodermal and mesodermal mesenchyme cells. The reaction was much strengthened when the mesenchyme concentrated to form the cartilage. The cutis layer and the anterior segments of the myotome, and all the mesodermal mesenchyme revealed intensive phosphatase activity. Prior to the differentiation of the heart, the anterior parts of the ventral mesoderm which seemed to be the presumptive material of the heart, reacted actively and with differentiation of endothelium and pericardium the enzyme activity tended to increase. In the later stage, the differentiating eye, lens, liver, foregut, anterior part of the lateral plate, a small part of the notochord and neural tube reacted also weakly. On the other hand, the reaction was very weak or not detectable in the epidermis, neural tissues, notochord and most of the myotomes.

B. *Rhacophorus schlegelii*, var *arborea*

The eggs and embryos of this species are suited for the study of phosphatase cytochemistry due to the following three reasons: (1) As stated in the foregoing chapter the eggs and embryos are completely devoid of any observable pigment granules till shortly prior to hatching; (2) In comparison with the preceding species the enzymatic activity is much stronger and begins to appear in earlier stage; (3) This species has been fully investigated histochemically in our laboratory (SH-group of protein (NAKANE, 1947); ribonucleic acid, indophenol oxidase, ascorbic acid, acetalphosphatide (TAKADA, 1950) some basic amino acids (OSAWA, unpublished)).

The section was incubated for 30 min., 1, 2, 3, 4 or 5 hrs., in particular cases also for 24 hrs. In some experiments the glycerophosphate was substituted by yeast ribonucleic acid (MERCK) but the pattern obtained was closely similar to that of the former, although resulted color was somewhat darker in comparison with the case of glycerophosphate.

The precipitation caused by GOMORI's technique was always found in both matrix and yolk granules in the cytoplasm, while the nucleus did not show any appreciable deposit at least until relatively later stage. However in the advanced embryos, nuclear staining was clearly observed in some tissues such as mesenchyme, and retina. But as to the intracellular localization of the enzyme activity one must be cautious to draw conclusion from such observation alone.

(a) St. 0 (uncleaved fertilized egg); St. 6 (morula stage): No reaction was obtained even after 24 hrs. of incubation.

(b) St. 8 (early gastrula): In some sections a very feeble reaction was obtained in the marginal zone. In another section however no reaction was found. Even in the positive case the site of the reaction appeared irregularly scattered.

(c) St. 13 (late gastrula stage): Marked synthesis of the enzyme appeared to occur during the process of gastrulation. Relatively definite localization of the activity was observed in the embryos of this stage. The enzyme activity was rather strong in the invaginating and invaginated lateral and ventral mesoderm and in the antero-ventral wall of the archenteron and possibly also in its adjacent mesoderm. Therefore in the longitudinal section (text-fig. 5), the

Text-fig. 5. Localization of the alkaline phosphatase activity in the late gastrula embryo of *Rhacophorus schlegelii*, var. *arborea*.

reaction was observed in the dorsal lip of blastopore and it weakened gradually towards the anterior end along the dorsal roof of the archenteron, but it strengthened again markedly at the antero-ventral wall of the archenteron. The ventral lip of blastopore reacted as strong as the antero-ventral wall of the archenteron. On the other hand, in the cross section the reaction was found rather strong in the lateral mesoderm, and very feeble or negative in the presumptive notochord and somite.

The lateral and ventral periphery of the endodermal yolk mass also reacted weakly. Both the presumptive epidermis and presumptive neural plate did not revealed any appreciable enzymatic activity after 5 hrs. of incubation.

(d) St. 22 (neurula) (pl. 1, fig. 4, 5): The general pattern remained as in the late gastrula, although the intensity was markedly increased. The epidermis was negative throughout. No activity could be detected also in the neural plate, excepting the following part: weak but distinct reaction could be observed in the contact surface of the neural plate with anterior part of the presumptive notochord. The dorsal mesoderm (notochord and presumptive somite) was devoid of reaction excepting few anterior somites which showed weak enzyme activity. The lateral mesoderm always revealed relatively intensive precipitation throughout the body (pl. 1, fig. 5). The antero-ventral wall of the archenteron (probably including some part of adjacent mesoderm) showed marked reaction in wide range (pl. 1, fig. 4). The peripheral zone of the endodermal yolk mass showed considerable reaction, the lateral part just under the lateral mesoderm being most intensive, the ventral part free from the mesoderm being feeble, and the dorsal part being devoid of reaction (pl. 1, fig. 5).

In order to check the possible false localization due to translocation of enzyme or/and calcium phosphate during fixation and incubation following series of experiments were made: (1) To avoid the diffusion of enzyme during fixation, the material was fixed and imbedded by the aid of freezing-drying apparatus. The paraffin section was then incubated in the GOMORI's medium for 5 hrs. The experimental section was compared with control, fixed by acetone according to the routine technique. As a result, there existed no essential difference between two slides, but in the former the reaction was much stronger as a whole and some reaction was observed also in the epidermis where no activity was found when fixed in acetone. Therefore, it may be concluded that no appreciable enzyme diffusion probably occurs at least in the course of acetone fixation. (2) To check diffusion of the enzyme or/and of the calcium phosphate during incubation, the following operative isolation was carried out. Acetone fixed section was attached to the slide. Each region which should reveal distinct silver precipitation in the total section was isolated completely by the aid of fine needle from the rest part of the section. Each operated sec-

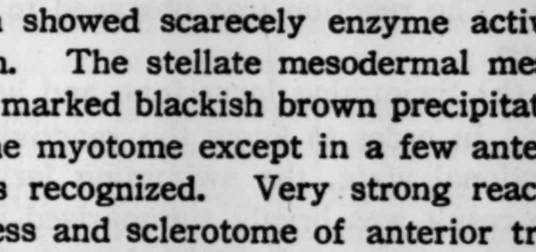
tion was incubated separately in the substrate solution for 5 hrs. Thus the anterior end of the endoderm, latero-ventral mesoderm, peripheral region of endoderm and some part of neural plate (see above) and anterior part of somite were isolated. The result obtained was thoroughly comparable with that of non-operated, total section. The fact shows absence of an observable diffusion during 5 hrs. incubation. Thus the *intercellular* pattern of the enzyme activity obtained in total section seems to be true at least in the neurula stage.

(e) St. 31 (tail bud stage, text-fig. 6) (pl. 1, fig. 7): Neural tube have very feeble reaction throughout the embryo, the ventral half of the neural tube reacting slightly stronger than the dorsal half. In this stage the optic cup was being formed from the wall of the forebrain. The optic cup and optic stalk revealed strong blackish brown precipitation (pl. 1, fig. 7). As no reaction was observed in earlier stages in this region, the fact might suggest that the alkaline phosphatase was rapidly synthesized during the morphogenesis of the regions occured. The epidermis was always negative. The mesectodermal mesenchyme, which retained the spherical form showed scarecely enzyme activity. The notochord did not show any coloration. The stellate mesodermal mesenchyme cells of the anterior levels revealed marked blackish brown precipitation. In the somite, there was no activity in the myotome except in a few anterior segments where considerable reaction was recognized. Very strong reaction was found in the cutis layer, ventral process and sclerotome of anterior trunk somite, decreasing towards posterior. The pronephros which was still non-tubular, reacted strongly. In the lateral plate, the reaction was rather intensive in the anterior region, but not found in the posterior region.

Text-fig. 6. *Rhacophorus schlegelii*, var. *arborea*, St. 31 (tail bud stage).

The antero-ventral wall of the archenteron showed very intensive reaction in wide range. Throughout the endodermal yolk mass, a moderate reaction was found, which showed a gradient of intensity from periphery (particularly from the ventral) to centre: after 30 min. to 1 hr. of incubation, the activity was limited in the lateral and ventral periphery of the yolk mass, but no precipitation was observed in the internal part. After 2∼5 hrs. of incubation, however, besides the peripheral region, also the internal region attained relatively marked coloration. It may be recalled that in the foregoing stages, even after 5 hrs. of incubation only the peripheral region of the yolk mass showed the reaction.

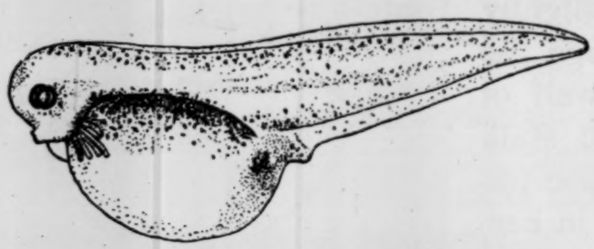
(f) St. 44 (embryo before hatching, text-fig. 7) (pl. 2, fig. 8, 9, 10, 11): The basic pattern of the distribution of the enzyme seemed to be unaltered, although the intensity increased. Pronephros (pl. 2, fig. 10), heart (pl. 2, fig. 11), stellate mesenchyme cells (pl. 2, fig. 9, 10) as well



Text-fig. 7. *Rhacophorus schlegelii*, var. *arborea*, St. 44 (embryo before hatching).

as the condensed mesenchyme cells (pl. 2, fig. 8) reacted intensively. In the pronephros the enzyme was found concentrated on the internal surface of the lumen. In general the latero-ventral mesoderm reacted considerably but thin layer of the mesoderm around the endodermal yolk mass gave a weak coloration at the middle level of the body. The reactivity of the endodermal yolk mass increased markedly in comparison with St. 31 (pl. 2, fig. 10) but the gradient of intensity was unchanged. The vacuolized notochord, and myotome showed no sign of staining, or at most a very weak staining (pl. 2, fig. 10).

(g) St. 47 (newly hatched embryo, text-fig. 8): The difference from the preceding stage was as follows: with progress of differentiation, retinal reactivity decreased. In this stage the reaction was rather stronger in the iris, than in the retina proper. The epidermis throughout the body was lined by the mesoderm (corium) which had strong activity. The dorsal part of the endodermal yolk mass, particularly around the digestive tube appeared to have considerable precipitation. The reaction was observed to concentrate on the surface of the digestive lumen.



Text-fig. 8. *Rhacophorus schlegelii*, var. *arborea*, St. 47 (newly hatched embryo).

(h) Swimming larva (one and half months after hatching) (pl. 2, fig. 12, 13, 14): In order to know the reactivity of each already differentiated organ in functional state, the swimming larva was put on test in total serial section. The result was briefly summarized in the Table II. The detailed localization in each organ need not be described here.

Table II. Relative intensity of alkaline phosphatase activity of some organs and tissues of the swimming larva of *Rhacophorus schlegelii*, var. *arborea*. Absence of colour is indicated by −, light brown, brown, blackish brown and solid black by +, ++, +++ and ++++ respectively.

Incubation period → / Organ ↓	1 hr.	3 hr.
Brain and nerve cord	+	++ ~ +++
Heart [1]	+++	++++
Intestine	+++	++++
Liver	+	++
Kidney [2]	++++	++++
Cartilage [3]	−	−
Retina [3]	− ~ +	− ~ +
Cornea [3]	−	+
Lens [3]	−	−
Muscle [3]	− ~ +	− ~ +
Notochord	−	−
Blood cells	−	−

Ref.: [1] pl. 2, fig. 13; [2] pl. 2, fig. 14; [3] pl. 2, fig. 12.

DISCUSSION

a. *Technical problems.* In this report, the validity of the cytochemical method of alkaline phosphatase proposed by TAKAMATSU (1938, 1939) and Go-

MORI (1939) is tested by applying it on the amphibian oocytes and embryos. The results obtained indicate that the clear staining after longer incubation periods on the nucleoli, nuclear sap of the young oocytes and on the yolk granules of the grown ones is not caused by the enzyme activity *in situ*, but represents an artefact due to the enzyme or/and calcium phosphate diffusion from the follicle, the site having a high phosphatase activity. The possibility that the artefact is caused by the enzyme diffusion during fixation can be excluded at least for the case of oocytes here studied, because the nucleoli, nuclear sap and yolk granules do not stain when the follicle is removed after fixation. Considered from the results here presented and that of earlier workers, it is probable that the calcium phosphate or/and the enzyme diffuse out from the follicle, and the diffusing substance is taken up selectively by nucleoli, nuclear sap and yolk granules. GOMORI (1950 b) stated that the diffusion did not occur in the substrate medium above pH 9.2, but our results and those of JACOBY and MARTIN (1949), and of NOVIKOFF (1951) contradict this opinion. That both calcium phosphate and the pure alkaline phosphatase are absorbed by some cell components especially by the nucleus is reported by NOVIKOFF (1951), GOMORI (1950 b), NEWMAN *et al.* (1950) and YOKOYAMA *et al.* (1950). However we have no conclusive data[4] to decide whether the diffusing substance during the course of incubation should be identified as the enzyme itself or as calcium phosphate, or as both of them.

It is known that the diffusing substance(s) is specifically absorbed by the nucleic acids-containing cell structures such as chromosome, nucleolus and the desoxyribonucleic acid-rich band of the salivary gland chromosomes of *Drosophila* (*cf.* NOVIKOFF, 1951). Cytochemical study of the amphibian oogenesis shows that ribonucleic acid is rich in the nucleoli, the nuclear sap and the cytoplasm of the young oocytes, but very poor in the same structures of the grown oocytes (OSAWA, unpublished data; see also BRACHET (1947 a)). Nucleic acid-containing nucleoli and nuclear sap of the young oocytes have the capacity of absorbing diffusing substance, but the nucleic acid-free yolk granules also show the capacity, while the nucleic acid-rich cytoplasm of the young oocytes hardly takes up the substance. These facts indicate that the observed artefact does not simply depend on nucleic acid content of the structure. In fact, NOVIKOFF (1951) already showed that the sections treated both with ribonuclease and desoxyribonuclease still have the affinity.

On the other hand, it was clearly showed that, in the neurula, the regional difference of the silver precipitation represents probably the true distribution of the enzyme activity within the embryo. However this does not necessarily mean that also intracellular location of the precipitation can be trusted in this case.

b. *Oogenesis.* The histochemical studies reported in this paper shows that during the amphibian oogenesis the alkaline phosphatase activity is present

[4] In her recent publication which appeared after the completion of this paper, MOOG (1951), reported that the *enzyme* in fixed and mounted section of the duodenum does not diffuse during short incubation (1½ hrs.).

only in the egg follicle, but probably not in the egg cell proper. KRUGELIS (1947) observed that after long incubation time "this enzyme" was present in the nuclear sap, nucleoli and chromosomes of the young oocyte, in which active synthesis of yolk protein was assumed to occur. Later on, in the full grown oocytes, the reaction became much weaker in the nuclear sap. The picture in the nucleus is closely similar to that of artefact above reported. Thus, the positive reaction observed by KRUGELIS in the nucleoli and nuclear sap might be only a secondary reaction caused by diffusion.[5] The presence of phosphatase in the nucleoli is reported in various invertebrate oocytes (sea-urchin, *Paracentrotus lividus*—WICKLUND, 1948; spider, *Tegenaria domesticus*—BRADFIELD, 1949; milleped, *Glomeris marginata*—FAURÉ-FREMIET, COURTINES et MUGARD, 1950; *Limnaea stagnalis*—ARVY, 1945; etc.). It is worthwhile to reexamine whether the alkaline phosphatase is indeed present in nucleoli of the oocytes in general, or the observed precipitation is nothing but an artefact as it is the case in amphibian oocyte.

Citing KRUGELIS' data, BRACHET (1947) stated that alkaline phosphatase in the nucleus might play a role in the course of yolk protein synthesis. The view is not supported by our data. But it may be not appropriate to deny existence of phosphatase altogether in the germinal vesicle from the histochemical study alone. In fact BRACHET (1944) found this enzyme, though in small amount, in the isolated germinal vesicle of the oocyte by quantitative estimation. In spite of the suggested cooperation of alkaline phosphatase and ribonucleic acid (*cf.* DAVIDSON, 1949), we cannot establish a parallel relationship, at least as to location, between both substances.

It is well known that the intestinal and kidney phosphatase participates in the resorption and reabsorption of glucose respectively (*cf.* LIPMANN, 1941). Thus the alkaline phosphatase of the egg follicle may be supposed to take part in the similar mechanism, *i.e.* absorption of nutrition for oocytes.

c. *Embryogenesis.* As shown in the preceding chapter, alkaline phosphatase activity develops more later in *Hynobius* than in *Rhacophorus*, but the pattern of distribution and the behaviour is essentially the same within both species. Therefore the result obtained for *Rhacophorus* will be here discussed.

The alkaline phosphatase is not detectable with our histochemical technique during the cleavage, begins to show its activity in the early gastrula, and increases progressively with development. This tendency conformes well with the data obtained by KRUGELIS (1950) with quantitative method.

With regard to the localization, however, the result here obtained is not in agreement with that of KRUGELIS (1947)(*cf.* introduction). It is not clear at the present time, whether the inagreement depends upon difference of technique or of the material used.

KRUGELIS (1950) estimated the alkaline phosphatase activity per embryo

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[5] The study shows further that the cobalt visualization technique of GOMORI (1941) which KRUGELIS adopted in her study (personal communication to Dr. YAMADA) is not adequate for the amphibian embryos as well as for the oocytes, since yolk granules and nucleoli have strong affinity to cobalt which causes non-enzymatic cobalt sulphide precipitation.

during development of *Xenopus* and *Amblystoma* by the aid of micro-photometric technique, and confirmed that the enzyme activity increased steadily during development. The curve presented by KRUGELIS shows the very low enzyme activity in the early cleavage stage and its considerable increase after the onset of gastrulation.

Moreover, KRUGELIS (1950) and GREGG and LØVTROP (1950) using the same method, showed that there exists a clear animal-vegetative gradient as to the enzyme content in the early gastrula stage.

The discrepancy between the biochemical data and ours may be interpreted by making the following assumption. The weak alkaline phosphatase activity, which exists until gastrulation, can not be visualized by means of our histochemical technique, only the fraction of the enzyme which increases after the gastrulation being detectable by our method.

In the following the significance of the enzyme for the developmental process in each organ will be considered. Table II shows the relative intensity of the alkaline phosphatase activity in some tissues of the free swimming larva of *Rhacophorus*. In this case, all organs described in the table apparently function and the alkaline phosphatase detected histochemically probably concerns with the function of each organ. From this table, it is obvious that the kidney and the heart contain a large amount of alkaline phosphatase. In the neurula stage (probably also in the late gastrula stage), the presumptive pronephric region and the presumptive heart apparently contain considerable amount of the enzyme, and this increases steadily with differentiation of the region. And in the developing digestive tube the activity attains high level in relatively later stage, the functional intestinal epithelium containing a large amount of the enzyme. It may be thus assumed that the growing amount of the enzyme detected during the embryogenesis of above three organs represents the course of the gradual synthesis and accumulation of the enzyme *in situ*, which is destined to fulfill the function of the organ after the completion of the embryogenesis (the functional differentiation of the organ).

The examples of the enzyme systems which show a similar behaviour in the course of development were reported by SAWYER (1943) and MOOG (1950) who studied the development of cholinesterase in the neuromuscular system of the amphibia and alkaline phosphatase in the duodenum of the chick respectively.

It is interesting to note that while the eye vesicle in the early embryonic stage contains a large amount of the enzyme, the functional retina shows only very poor reaction for the phosphatase test. Thus the phosphatase of the differentiating retina might be supposed to concern with histogenesis of the retina. Essentially the same result was obtained in the chick embryo both with histochemical and biochemical method (MOOG, 1944; LINDEMAN, 1949). The finding seems to be of importance from the stand point of embryology: Inspite of pronounced morphogenetic differentiation of the neural plate derivatives during the early embryogenesis, till now there have been no direct data which indicate the regional chemical difference within the early neural system. Clear localization of the alkaline phosphatase activity in the optic vesicle shows however, that the biochemical process occurring in this district certainly differs

from other regions of the neural system. But this enzyme activity cannot be the cause of the determination of optic rudiment, because the determination of the rudiment occurs already during the last phase of gastrulation, when no enzyme activity can be detected in its presumptive region with our method.

All the mesenchyme cells show very strong activity, in their free stellate condition as well as in the condensed state during the cartilage formation, but the differentiated cartilage does not react to the phosphatase test. Here the phosphatase seems to relate to the chondrogenesis itself. Also for the alkaline phosphatase found in the anlagen of the kidney, heart and digestive tube a histogenetic significance might not be denied besides the functional one.

The behaviour of the enzyme in the endodermal yolk mass is very important from the biochemical stand point of yolk decomposition. The activity is weak in the gastrula and neurula, and is restricted only in its peripheral region. The reaction is more and more intensified with the progress of development, showing the strong activity in its central part before and after hatching. It might be added that not only in the endoderm, but also in others part of the embryo such as in retina, mesenchyme etc., a part of alkaline phosphatase might relate to the yolk utilization of the organ rudiment.

On the other hand, muscle, epidermis, notochord and lens have very weak or no activity throughout entire periods.

Summarizing what stated there may be distinguished the following groups of organs according to the behaviour of the phosphatase activity: (1) Enzyme activity appears in the primordia and increases progressively during differentiation and has a definite functional significance in the completed organ (kidney, heart, digestive tube and probably brain and nerve cord). (2) The phosphatase appears in the course of differentiation and later disappears when differentiation ceases (eye, cartilage and endodermal yolk mass). (3) The enzyme is very poor or lacking altogether during the entire course of development (muscle, epidermis, notochord and lens).

The close relation between alkaline phosphatase and ribonucleic acid-containing granule, and the presence of the alkaline phosphatase in the nucleus during early amphibian and chicken development reported by BRACHET (1946, 1947b, 1949, 1950) and KRUGELIS (1947), and MOOG (1944) can not be maintained by our data.

According to TAKADA (unpublished data), the differentiating amphibian retina contains a large amount of ribonucleic acid but the adult one has no observable basophila. This picture seems to be similar to that of alkaline phosphatase. However it is unlikely that relationship of alkaline phosphatase with nucleic acids is a general phenomenon during development. Moreover, as to the nuclear phosphatase we could not show the activity in the nucleus in the early developmental stage. KRUGELIS', BRACHET's and also MOOG's results must be reexamined critically considering the diffusion artefact during incubation periods.

BRADFIELD (1946, 1949) and JEENER (1948) stressed the indispensable role of alkaline phosphatase during fibrous protein synthesis. MINGANTI (1950) emphasized close relationship of alkaline phosphatase with synthesis of fibrous

protein during the development of *Limnaea.* But the alkaline phosphatase activity is very low or completely negative in the entire period of the differentiation of myotome, in which synthesis of typical fibrous proteins does occur.

SUMMARY

1. By means of the method of TAKAMATSU and GOMORI, localization of the alkaline phosphatase was studied in the oocytes of *Triturus pyrrhogaster,* and in the early developmental stages of *Rhacophorus schlegelii,* var. *arborea* and *Hynobius tokyoensis.*

2. It was confirmed that the artefact due to the diffusion of the enzyme or/and reaction product occurs during incubation time. Some supplementary experiments to determine the sites of *phosphatase activity* in the oocytes and the embryos were carried out.

3. In all stages of oocytes, only the egg follicle contains alkaline phosphatase, the egg cell proper being probably devoid of the enzyme in so far as detectable with the technique here employed. The supplementary experiments showed decisively that the intensive staining in the nucleoli, nuclear sap of the young oocytes and yolk granules of the grown oocytes obtained with ordinary technique is nothing but an artefact caused by diffusion.

4. During the development of *Rhacophorus,* the alkaline phosphatase was found mainly in the following organs and their rudiments: kidney, heart, digestive tube, nervous system, retina, mesenchyme and endodermal yolk mass. The changes of the enzyme activity during the course of development were described for above mentioned organs. Muscle, epidermis, notochord and lens have very weak or no activity throughout entire periods.

5. Alkaline phosphatase begins to be detectable later in *Hynobius* than in *Rhacophorus,* but the pattern of distribution and behaviour is essentially the same for both species.

6. Parallel distribution of alkaline phosphatase and of ribonucleic acid in the early phase of development as insisted by earlier writers could not be evidenced. Moreover no indication was obtained which speaks for the presence of the enzyme within the nucleus in the earlier phase of development.

7. The possible biological meanings of the alkaline phosphatase during oogenesis and embryogenesis were discussed.

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EXPLANATION OF FIGURES

Staining of the amphibian oocytes and embryos by means of TAKAMATSU-GOMORI's alkaline phosphatase technique.

Plate 1.

Fig. 1. *Triturus* oocytes. Only the follicle is stained. Left below, grown oocyte. Black appearance in the egg is due to pigment granules but not to the reaction. 1 hr. incubation.

Fig. 2. The same as above. 24 hrs. incubation. Beside the follicle, nucleoli, nuclear sap of the young oocytes and yolk granules of the grown ones are stained positively.

Fig. 3. Young oocytes of *Triturus*. Heat-inactivated section attached face to face with another section having the enzyme activity and incubated in the substrate medium. Note clear staining of nucleoli and nuclear sap, but almost no coloration of follicle. 24 hrs. incubation.

Fig. 4. *Rhacophorus* neurula. Frontal part of the embryo cut transversely. Mesoderm (particulary lateral) and endoderm are positive. Neural plate, epidermis and mesectoderm have no coloration. 3 hrs. incubation (freezing-drying).

Fig. 5. *Rhacophorus* neurula. Middle part of the same embryo as above. Lateral mesoderm and peripheral region of the endoderm are clearly stained, some coloration also in the epidermis. Note the absence of reaction in the nuclei. 3 hrs. incubation (freezing-drying).

Fig. 6. *Rhacophorus* early tail bud stage. (This stage is not described in the text, as it was employed only in the preliminary experiments). Almost the same pattern as fig. 5. 3 hrs. incubation.

Fig. 7. *Rhacophorus* tail bud stage (somewhat advanced in comparison with above). Optic vesicle, optic stalk and heart rudiment are strongly stained. 3 hrs. incubation.

Plate 2.

Fig. 8. *Rhacophorus* embryo before hatching. Differentiating eye and chondrogenetic mesenchyme (ventral to the eye) are very strongly stained, while the induced lens and the brain have almost no coloration. 3 hrs. incubation.

Fig. 9. *Rhacophorus* embryo before hatching. Free stellate mesenchyme cells show strong reaction. Neural tube, myotome and notochord are only weakly stained. 3 hrs. incubation.

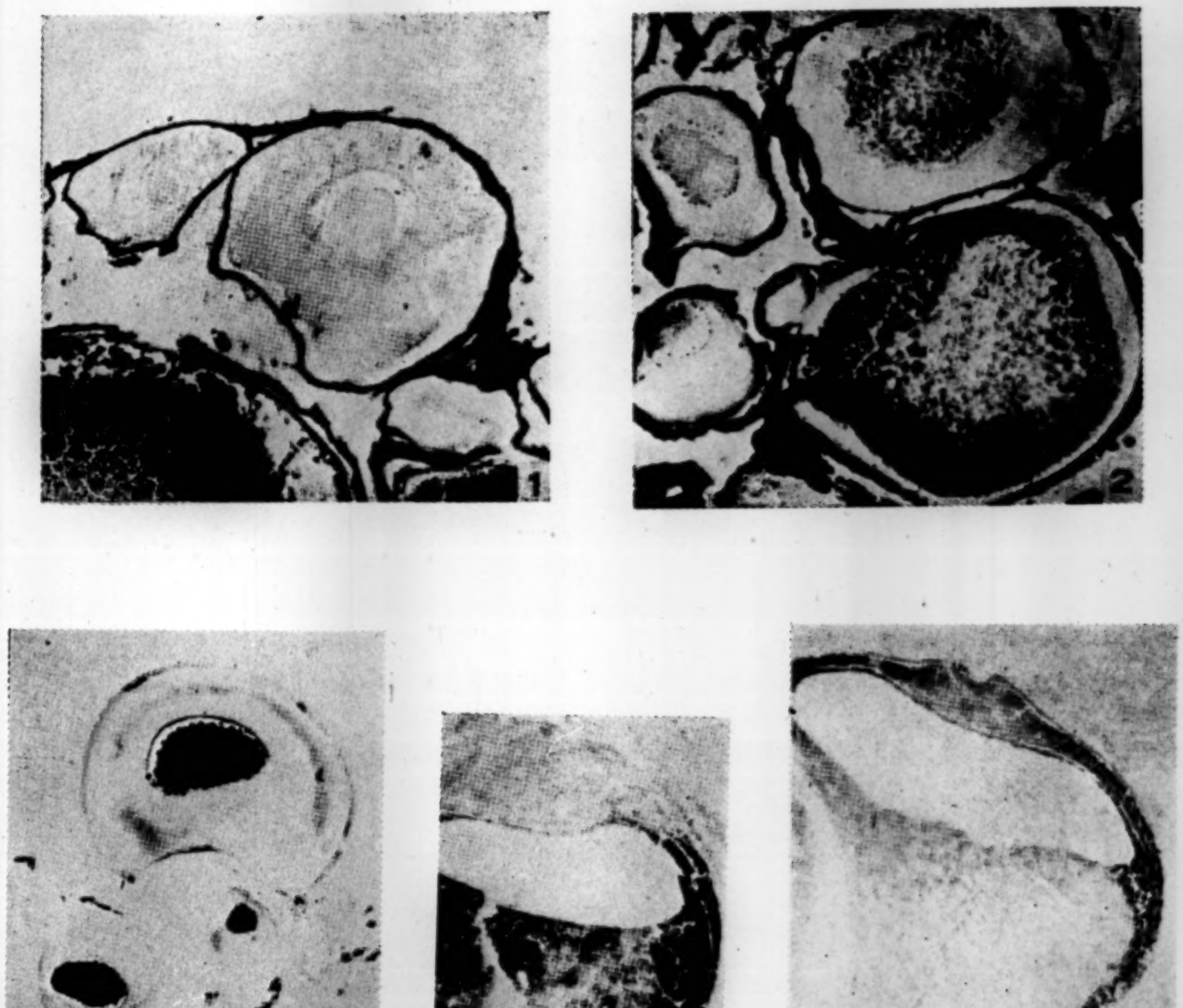
Fig. 10. *Rhacophorus* embryo before hatching. Differentiating heart having strong enzyme activity. 3 hrs. incubation.

Fig. 11. Swimming larva of *Rhacophorus* (one and half months after hatching). Differentiated retina showing feeble reaction and differentiated cartilage without activity. Compare with differentiating eye and cartilage respectively (figs. 7, 8 and 9). 3 hrs. incubation.

Fig. 12. Swimming larva of *Rhacophorus*. Differentiated heart possessing strong activity. 3 hrs. incubation.

Fig. 13. Swimming larva of *Rhacophorus*. Differentiated kidney having intensive enzyme activity. 3 hrs. incubation.

PLATE 1

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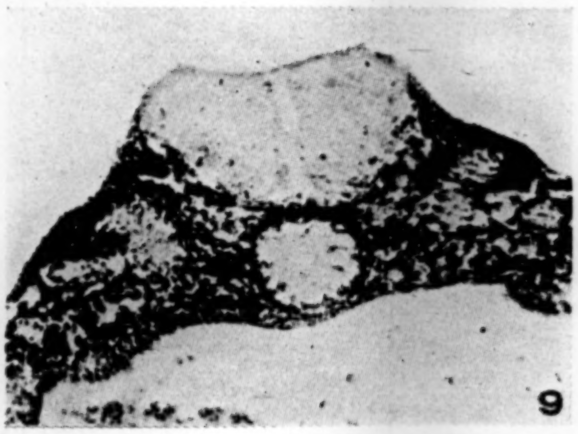
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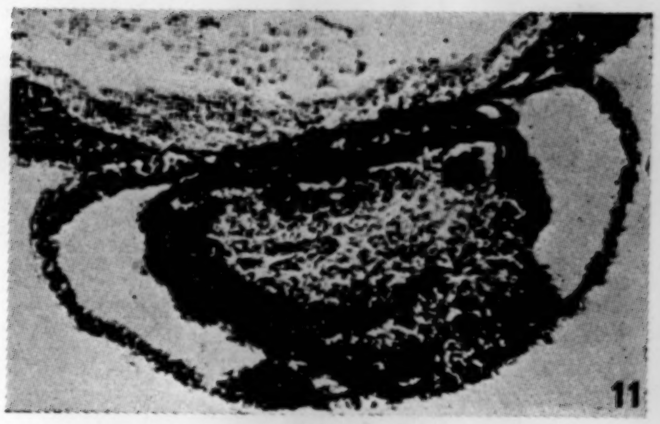
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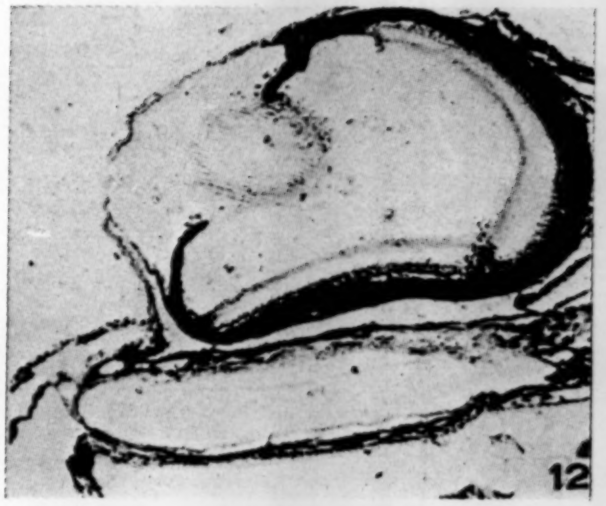
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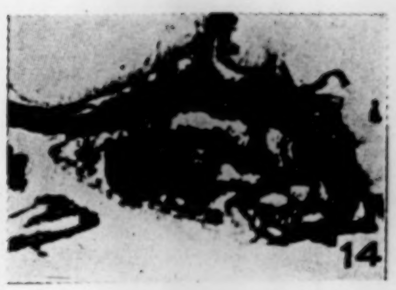
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